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Properties of Fiber Cell Plasma Membranes Isolated from the Cortex and Nucleus of the Porcine Eye Lens

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Abstract

The organization and physical properties of the lipid bilayer portion of intact cortical and nuclear fiber cell plasma membranes isolated from the eyes lenses of two-year-old pigs were studied using electron paramagnetic resonance (EPR) spin-labeling. Membrane fluidity, hydrophobicity, and the oxygen transport parameter (OTP) were assessed from the EPR spectra of precisely positioned spin labels. Intact cortical and nuclear membranes, which include membrane proteins, were found to contain three distinct lipid environments. These lipid environments were termed the *bulk lipid domain*, *boundary lipid domain*, and *trapped lipid domain* (lipids in protein aggregates). The amount of boundary and trapped lipids was greater in intact nuclear membranes than in cortical membranes. The properties of intact membranes were compared with the organization and properties of lens lipid membranes made of the total lipid extracts from the lens cortex or nucleus. In cortical lens lipid membranes, only one homogenous environment was detected, which was designated as a bulk lipid domain (phospholipid bilayer saturated with cholesterol). Lens lipid membranes prepared from the lens nucleus possessed two domains, assigned as a bulk lipid domain and a cholesterol bilayer domain (CBD). In intact nuclear membranes, it was difficult to discriminate the CBD, which was clearly detected in nuclear lens lipid membranes because the OTP measured in the CBD is the same as in the domain formed by trapped lipids. The two domains unique to intact membranes—namely, the domain formed by boundary lipids and the domain formed by trapped lipids—were most likely formed due to the presence of membrane proteins. It is concluded that formation of rigid and practically impermeable domains is enhanced in the lens nucleus, indicating changes in membrane composition that may help to maintain low oxygen concentration in this lens region.

Keywords

cholesterol; membrane domains; fluidity; hydrophobic barrier; oxygen permeation; EPR; spin labeling

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1. Introduction

Good vision requires a transparent lens with minimal light scattering. Because the lens is avascular, it is necessary that nutrients (including oxygen) must diffuse into its interior. The loss of subcellular organelles during maturation of fiber cells reduces light scattering, and, thus, the plasma membrane essentially becomes the only membranous structure in mature fiber cells (Beebe, 2003). For light and nutrients to reach the center of the lens, they must pass through thousands of fiber cell membranes. Therefore, the fiber cell plasma membrane is a significant structure that can affect the function of the entire lens. The properties of fiber cell membranes are largely determined by two components: (1) membrane proteins, which allow communication between layers of fiber cells and active transport of water, ions, and other small molecules less than about 1 kDa (White and Bruzzone, 2000), and (2) the lipid bilayer portion of the membrane, which determines bulk membrane properties (including diffusion barriers) and can affect the properties of membrane proteins (Borchman and Yappert, 2010; Subczynski et al., 2012).

The goal of this research was to study the properties of the lipid bilayer portion of the fiber cell membrane. The first aim examines lens lipid membranes (without proteins) made of the total lipid extract from fiber cell plasma membranes. The second aim looks at intact fiber cell plasma membranes (containing membrane proteins) isolated from different regions of the lens. The evaluation of lens lipid membranes and the lipid bilayer portion of intact lens membranes is important in order to establish the response of spin labels to lipid-protein interactions. With this information, we can study the effects of changes in lipid composition, as well as protein/lipid ratio change with age (Broekhuysen and Kuhlmann, 1974, 1978; Deeley et al., 2008; Gooden et al., 1982; Yappert et al., 2003), regional differences in fiber cell membranes (Jacob et al., 1999; Yappert et al., 2003), and differences among species (Broekhuysen and Kuhlmann, 1974, 1978; Deeley et al., 2008; Gooden et al., 1982; Yappert et al., 2003). For example, changes that occur with age include depletion of glycerophospholipids and increased amounts of sphingomyelins, with preferential enlargement in dihydrosphingomyelin in humans (Borchman and Yappert, 2010). Phosphatidylcholine is the major phospholipid in the lens membranes of animals with short life spans, while sphingomyelin is the predominant phospholipid in the lens membranes of animals with long life spans, including humans (Borchman et al., 2004; Deeley et al., 2008). Interestingly, a highly saturated sphingolipid content is concomitant with a high amount of cholesterol (Rujoi et al., 2003), which not only saturates the phospholipid bilayer but also leads to the formation of cholesterol bilayer domains (CBDs¹) within these membranes (Raguz et al., 2008; Raguz et al., 2009). Changes in cholesterol content in the fiber cell plasma membrane that occur with aging are indicated by an increase in the total cholesterol/phospholipid (Chol/PL) molar ratio. There is a higher Chol/PL molar ratio in the nucleus than in the cortex (Borchman et al., 1989; Fleschner and Cenedella, 1991; Li and So, 1987; Raguz et al., 2009; Rujoi et al., 2003). Such great variation in phospholipid composition and cholesterol content with age and lens region suggests that unique mechanisms exist to maintain homeostasis in the fiber cell membrane. This observation is especially significant for humans. Among mammalian lenses, those from humans have the longest life span, and changes in their lens lipid composition with age are the most pronounced (Li et al., 1985, 1987; Yappert et al., 2003). The fiber cells of human lenses do not regenerate, and cells located in the nucleus of an adult human lens are as old as the individual.

Membrane proteins that perform several functions in young human lenses perform the same functions in older lenses, which have altered phospholipid compositions. Additionally, the amount of proteins in fiber cell plasma membranes increases with age, and intact nuclear membranes are denser than cortical membranes in membrane proteins (Jacob et al., 1999).

Compared to most types of plasma membranes, there are only a limited number of fiber cell integral transmembrane proteins (see review by (Bassnett et al., 2011)). They can be separated into two major classes of integral membrane proteins. The first class is formed by aquaporin-0 (AQP0; formerly, MIP or MIP26), which is the most abundant protein in the lens fiber cell membrane and is a member of the water transport family of integral channel proteins (Agre, 2004). The second class is formed by connexins, Cx46 and Cx50, that form the connexin hemi-channels, with Cx46 found mainly in the cortex and outer nuclear layers and Cx50 found mainly in the nuclear core (Chung et al., 2007; Tenbroek et al., 1992; White et al., 1998).

The lipid composition of the lens fiber cell membrane changes as both animals and humans age (Deeley et al., 2008; Yappert et al., 2003). Usually, such notable changes would result in alteration of the physical properties of the membrane, which would then affect the function of proteins immersed in the lipid bilayer (Epand, 2005). Based on results obtained for lens lipid membranes (Mainali et al., 2011b; Raguz et al., 2008; Raguz et al., 2009; Widomska et al., 2007a; Widomska et al., 2007b) and simple models of lens lipid membranes (Mainali et al., 2011b; Mainali et al., 2011c, 2012; Raguz et al., 2011a, b), we have concluded that the saturating cholesterol content in fiber cell membranes keeps the bulk physical properties of lens lipid membranes consistent and independent of changes in phospholipid composition. Thus, CBDs, which ensure that the surrounding phospholipid bilayer is saturated with cholesterol, help to maintain homeostasis in the lens membrane when its phospholipid composition changes significantly. Obtained data also indicate that the major permeability barrier to oxygen transport across the lens lipid membrane is located at the CBD (Mainali et al., 2011b; Raguz et al., 2008; Raguz et al., 2009), which helps to maintain a low oxygen concentration in the lens interior (especially in the nucleus of the human lens where the Chol/PL mole ratio is as high as 4 (Li et al., 1987)). Also, the CBD occupies a significant portion of the membrane surface in the nucleus. The lens lipid membrane forms a high permeability barrier to the nonspecific passage of polar molecules into and out of the fiber cell because of its high hydrophobicity and high rigidity barrier to the nonspecific passage of nonpolar molecules (like molecular oxygen). These conclusions and hypotheses, which are based on measurements on membranes without a protein component, were summarized in a recent review (Subczynski et al., 2012).

Because of its saturating cholesterol content, the surface of lens lipid membranes is smooth (Plesnar et al., 2012; Widomska et al., 2007a), which decreases light scattering and helps to

¹The CBD is different from the raft domain. The former is a pure cholesterol bilayer immersed in the bulk phospholipid-cholesterol membrane (phospholipid bilayer saturated with cholesterol, which is in the liquid-ordered phase) (Preston Mason et al., 2003; Raguz et al., 2011a, b). The latter is formed by the mixture of phospholipids (mainly sphingolipids) containing ~33 mol% cholesterol (de Almeida et al., 2003; Veatch and Keller, 2003)D. Raft is a liquid-ordered-phase domain immersed in the liquid-disordered bulk membrane (Edidin, 2003; Simons and Vaz, 2004). Rafts have specific physiological functions, such as signal transduction, protein sorting, and lipid trafficking (Brown and London, 1998; Simons and Ikonen, 1997), while the appearance of the CBD is usually a sign of pathology (Tulenkov et al., 1998). However, in the eye lens, CBDs play a positive physiological role, maintaining lens transparency (Borchman et al., 1996; Jacob et al., 2001; Preston Mason et al., 2003) and possibly protecting against cataract formation (Jacob et al., 1999; Preston Mason et al., 2003). The functions of cholesterol and CBDs specific to the fiber-cell plasma membrane are discussed in (Subczynski et al., 2012). It should be mentioned here that lipid rafts have been isolated from lens fiber-cell membranes using cold Triton X-100 extraction (Cenedella et al., 2007; Rujoi et al., 2003; Tong et al., 2009). Rujoi et al. (Rujoi et al., 2003) indicated that formation of raft domains in the eye lens depends on raft proteins and not on lipid composition. Results presented by Tong et al. (Tong et al., 2009) showed that the AQP0, Cx46, and Cx50 can be sorted in the plane of the lipid bilayer. After Triton X-100 extraction, Cx46 and Cx50 were located primarily in detergent-soluble membranes (thus, outside raft domains), whereas AQP0 was found in both detergent-resistant and detergent-soluble membranes (thus, distributed between raft domains and bulk lipids). The authors concluded that protein-lipid interactions modify lens-protein distribution as well as lipid organization. In a critical review, Munro (Munro, 2003) stated that in membranes overloaded with cholesterol all lipids should be in the liquid-ordered phase, and the entire membrane should be organized as in a raft domain. The results we obtained for lens lipid membranes completely confirmed this statement—showing that lens lipid membranes are in the liquid-ordered phase (Raguz et al., 2008; Raguz et al., 2009; Widomska et al., 2007a). Additionally, it was shown that in model membranes Triton X-100 promotes the formation of raft domains, which suggests that isolated detergent-resistant and detergent-soluble membranes do not reliably report on the organization of lipids and proteins in cell membranes (Heerklotz, 2002; Lichtenberg et al., 2005).

maintain lens transparency. It is likely that this beneficial effect of cholesterol on light scattering is compensated by the effect of proteins associated with the plasma membrane of fiber cells. It was shown that during aging, and in the case of cataract formation, the association of α -crystallin with the membrane increases, which is accompanied by increased light scattering (Boyle and Takemoto, 1996; Cenedella and Fleschner, 1992; Tang et al., 1999). *In vitro* binding studies have shown that the binding capacity of α -crystallin to lipids from older lenses increases with donor age (Grami et al., 2005). Thus, the interaction of α -crystallin with membranes may be related to cataractogenesis because the denatured α -crystallin binds more deeply in the membrane (Cobb and Petrash, 2002; Tang and Borchman, 1998).

In studies of intact membranes, we cannot use the series of spin-labeled phospholipids (Fig. 1) that were successfully applied to the study of lens lipid membranes to obtain detailed profiles of certain membrane properties (Mainali et al., 2011b; Raguz et al., 2008; Raguz et al., 2009; Widomska et al., 2007a; Widomska et al., 2007b). These spin-labeled phospholipids cannot be incorporated into the lipid bilayer portion of the lens lipid membrane without the use of damaging solvents. Fortunately, a similar series of stearic acid spin labels exists (Fig. 1) that allows us to locate the nitroxide moiety of a spin label at different depths in the lipid bilayer and to obtain relevant profiles of membrane properties across the lipid bilayer portion of intact fiber cell plasma membranes. These spin labels can be inserted into intact membranes from their dry film (Ligeza et al., 1998) and, thus, with a minimal disturbance to membrane structure. Spin-labeled cholesterol analogues, androstane spin label (ASL) and cholestane spin label (CSL) (Fig. 1), can be inserted into lens lipid membranes. However, only ASL can label intact membranes. The unique distribution of spin labels between domains in intact and lens lipid membranes allows us to discriminate certain membrane domains and to obtain information about their structure and dynamics. Figure 2 is a schematic drawing that illustrates the distribution of appropriate spin labels in lens lipid and intact membranes. This figure also provides the guideline for our experiments and interpretation of data.

2. Materials and Methods

2.1. Materials

One-palmitoyl-2-(n-doxylstearoyl)phosphatidylcholine spin labels (*n*-PC, *n* = 5, 7, 10, 12, 14, or 16) and tempocholine-1-palmitoyl-2-oleoylphosphatidic acid ester (T-PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Doxylstearic acid spin labels (*n*-SASL, *n* = 5, 7, 9, 10, 12, or 16) and cholesterol analogues (androstane spin label [ASL] and cholestane spin label [CSL]) were purchased from Molecular Probes (Eugene, OR). Other chemicals of at least reagent grade were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Isolation of intact membranes and total lipids from cortical and nuclear fiber cell membranes

Porcine eyes from two-year-old animals were obtained on the day of slaughter from Johnsonville Sausage, LLC (Watertown, WI). The eyes were dissected, and the lenses from about 100 eyes were collected. The cortical and nuclear regions of these lenses were separated based on differences in tissue consistency (Estrada and Yappert, 2004; Rujoi et al., 2003). Cortical and nuclear intact membranes were isolated from tissue based on minor modifications of the method developed by Bloemendal et al. (Bloemendal et al., 1972), as reported earlier (Cenedella and Fleschner, 1992; Chandrasekher and Cenedella, 1995; Lim et al., 2005). The cortical and nuclear tissues were homogenized separately, each in 25 mL of *buffer A* (5 mM Tris HCL, 5 mM EDTA, 5 mM EGTA, pH 8.0). The homogenate was centrifuged (29000 g, 20 min, 4°C). The pellet was washed five times with *buffer B* (5 mM

Tris HCL, 2 mM EDTA, 2 mM EGTA, pH 8.0) and recovered by centrifugation (29000 g, 20 min, 4°C). Each time special care was taken to produce a uniform suspension by repeatedly aspirating the solution through a syringe fitted with an 18-gauge needle. Finally, the pellet was washed and resuspended with *buffer C* (0.1 M borate, pH 9.5) and stored at -20°C. Parts of these suspensions were used to determine the protein/lipid ratio. For this purpose, the membrane suspensions were sonicated (SDS was added to 0.1%), and the Lowry method was used to determine protein content (Pearce BSA Protein Assay Kit, Thermo Scientific [Rockford, IL]). To measure the total lipids from the cortical and nuclear tissues, materials were extracted separately based on minor modification of the Folch procedure (Folch et al., 1957), which was described earlier (Mainali et al., 2011b; Raguz et al., 2008; Raguz et al., 2009). Extracts were stored at -20°C. The final protein/lipid ratio was four times higher in nuclear membranes compared to cortical membranes.

2.3. Preparation of samples for EPR measurements

2.3.1. Spin-labeling of intact membranes—Films of *n*-SASL or ASL were prepared on the bottom of a test tube by drying the appropriate amount of spin label in chloroform (usually ~20 μ L of 1 mM solution). Only one type of spin label was present in each sample. Intact membrane suspensions (~0.2 mL) were added to the test tubes and shaken for about two hours at room temperature. Control measurements demonstrated that this incubation time was sufficient to incorporate nearly all of the spin-label molecules into the membranes. Spin-label concentration was always lower than 1 mol% of the total lipids. Finally, membrane suspensions were centrifuged for a short time, and the loose pellet was transferred to a 0.6 mm i.d. capillary made of gas-permeable methylpentene polymer (TPX) and used for EPR measurements (Subczynski et al., 2005).

2.3.2. Spin-labeling of lens lipid membranes—The lipid bilayer membranes used in this work were multilamellar dispersions (multilamellar liposomes) made from the lipid extracts from either the porcine eye-lens cortex or nucleus and containing ~1 mol% spin label (*n*-PC, 9-SASL, ASL, or CSL). The membranes were prepared using the film deposition method described previously (Kusumi et al., 1986; Mainali et al., 2011c).

2.4. EPR measurements

To further increase the signal-to-noise ratio, samples in TPX capillaries were centrifuged as described in (Subczynski et al., 2005). Conventional EPR spectra were recorded with a Bruker EMX spectrometer equipped with temperature-control accessories. All spectra were obtained at 37°C with a modulation amplitude of 1.0 G and an incident microwave power of 5.0 mW. To assess fluidity, maximum splitting values were measured for each component directly from the EPR spectra, as indicated in Fig. 3. Samples were thoroughly deoxygenated, yielding correct EPR line shapes. Maximum splitting values were measured within the accuracy of ± 0.25 G. To measure hydrophobicity, the *z*-component of the hyperfine interaction tensor of the *n*-PC or *n*-SASL, A_z , was determined from the EPR spectra for samples frozen at -165°C and recorded with a modulation amplitude of 2.0 G and an incident microwave power of 2.0 mW (Subczynski et al., 1994). Values of $2A_z$ were measured within the accuracy of ± 0.25 G.

Spin-lattice relaxation times, T_{1s} , of spin labels were determined by analyzing the saturation recovery (SR) signal of the central line obtained by short-pulse SR EPR at X-band (Kawasaki et al., 2001; Subczynski et al., 1989; Yin and Subczynski, 1996). Accumulations of the decay signals were carried out with 2048 data points on each decay. For measurements of the oxygen transport parameter (OTP), the sample was equilibrated with the same gas that was used for temperature control (*i.e.*, a controlled mixture of nitrogen and dry air adjusted with flowmeters [Matheson Gas Products, model 7631H-604]) (Kusumi et

al., 1982; Subczynski et al., 2005; Subczynski et al., 1992a). SR signals were fitted by single- or double-exponential functions. The uncertainties in the measurements of decay time from the fits were usually less than 0.05%, whereas the decay times determined from sample to sample were within an accuracy of $\pm 3\%$ when a single-exponential fit was satisfactory and within an accuracy less than $\pm 5\%$ (and $\pm 10\%$ for longer and shorter recovery time constants) when a double-exponential fit was satisfactory.

3. Results

3.1. Conventional EPR indicates the presence of phospholipid domains in intact membranes

Membrane fluidity was measured at different locations (depths) within the intact cortical and nuclear membranes as well as in lens lipid membranes based on the location of the nitroxide moiety on the alkyl chains of the phospholipid or stearic acid (Fig. 1). Maximum splitting values obtained from the EPR spectra were used as a convenient parameter to monitor membrane fluidity (Fig. 3H). Smaller maximum-splitting values indicated greater membrane fluidity (Kusumi et al., 1986). A remarkable feature of EPR spectra from lens lipid membranes (both cortical and nuclear [Figs. 3A, C, E, G]) was that there was no indication of the presence of two components in the *n*-PC spectra. This is in agreement with our previous work and allows us to conclude that the entire bulk phospholipid-cholesterol portion of lens lipid membranes is in the liquid-ordered-like phase (Raguz et al., 2008; Raguz et al., 2009; Widomska et al., 2007a). The CBD, which can be formed in these membranes, can only be detected with cholesterol analogue spin labels (see Fig. 2 for spin-label distribution). The latter results are described in section 3.3 and indicate that the bulk phospholipid-cholesterol bilayer in lens lipid membranes is homogeneous.

Figures 3B, D, F, H show representative conventional EPR spectra for 5- and 12-SASL in cortical and nuclear intact membranes. Although the majority of *n*-SASL is incorporated in the membrane, a very small amount remains free in the surrounding buffer. The intensity of this component (component 3 in Figs. 3B, D, F, H) depends on the buffer amount and the position of the nitroxide moiety on the stearic acid. The greatest intensity is observed for 9-, 10-, and 12-SASL. In lens lipid membranes labeled with *n*-PC, as described above, the water component was absent (see Figs. 3A, C, E, G). This component of the spectra does not interfere with measurements of maximum splitting, thus permitting us to focus on components of spectra coming from spin labels in the lipid environment of intact membranes (components 1 and 2 in Figs. 3B, D, F, H). As can be seen in Figs. 3B, D, there is no indication of the presence of two components in the EPR spectra of 5-SASL in cortical and nuclear intact membranes. Similarly, EPR spectra of 7-, 9-, and 10-SASL were also one-component spectra (not shown). However, EPR spectra of 12-SASL (Figs. 3F, H) and 16-SASL (spectrum not shown) clearly show one weakly immobilized component (2) and one strongly immobilized component (1). These immobilized components were assigned to the bulk lipid domain and the boundary plus trapped lipid domain, respectively. The basis for these domain assignments is from the literature (see also section 3.3). Spectra from the intact membranes are similar to those from boundary lipids formed around monomers of integral membrane proteins, as reported by Jost et al. and Ryba et al. (Jost et al., 1973; Ryba et al., 1987), as well as to those from membranes reconstituted with monomers and aggregates of bacteriorhodopsin, as reported by Ashikawa et al. (Ashikawa et al., 1994).

Why is it that 5-, 7-, 9-, and 10-SASL do not detect two environments in intact membranes while 12- and 16-SASL do? The time frame for the conventional EPR spin-labeling method is 100 ps to 10 ns. In bulk lipids, spectra of 12- and 16-SASL are sharp, and in boundary lipids, they are broad. Thus, peaks used to measure maximum splitting are clearly distinguished. The outermost peaks in the spectra of 5-, 7-, 9-, and 10-SASL, which are used

to measure maximum splitting, were broad. Also, their positions were very close in bulk and boundary lipids, which makes discrimination of components very difficult. However, the discrimination by oxygen transport (DOT) method, with a time frame from 0.1 to 100 μ s, allowed us to discriminate domains in intact membranes with only the use of *n*-SASL (see sections 3.3 and 3.5).

Thus, the presence of proteins in intact membranes induced formation of a new lipid domain, which was manifested by the strongly immobilized signal in the EPR spectra of 12- and 16-SASL (component 1 in Figs. 3F, H) in addition to the weakly immobilized signal typical for 12- and 16-SASL in lens lipid membranes (component 2 in Figs. 3F, H). We refer to this new domain as a boundary lipid domain that is situated around integral membrane proteins and other strongly immobilized lipids, which we hypothesize are lipids trapped between protein molecules. The weakly immobilized signal (component 2) is the major component of spectra from cortical membranes. While in nuclear membranes, the major component is formed by the strongly immobilized signal (component 1). This observation suggests that the amount of boundary and trapped lipids is significantly greater in nuclear membranes than in cortical membranes. This finding is in agreement with our own studies, and data from the literature indicate that the protein/lipid ratio is higher in the nucleus than in the cortex (Jacob et al., 1999). Therefore, we conclude that the unresolved EPR spectra from 5-, 7-, 9-, and 10-SASL mainly come from bulk lipids in cortical membranes and from boundary and trapped lipids in nuclear membranes.

3.2. Conventional EPR spectra of ASL indicate the presence of a strongly immobilized environment for cholesterol molecules in intact nuclear membranes

ASL is a spin-labeled cholesterol analogue (Fig. 1) that determines the distribution and properties of cholesterol in intact membranes (see distribution of ASL in Fig. 2). ASL can be incorporated into intact membranes and yields one-component EPR spectra in cortical membranes (Fig. 4B) and two-component spectra in nuclear membranes (components 1 and 2 in Fig. 4D). ASL spectra from cortical and nuclear lens lipid membranes are one-component (Figs. 4A, C) and practically identical to the spectrum of cortical intact membranes. In cortical intact membranes, ASL cannot discriminate any lipid domain formed by the presence of integral proteins (showing only component 2). This is somewhat surprising because 12- and 16-SASL showed the presence of boundary and trapped lipids. However, when the amount of proteins increases in nuclear intact membranes, the strongly immobilized component in the EPR spectrum is clearly present (component 1 in Fig. 4D). We hypothesize that in nuclear intact membranes the immobilized component is formed by ASL molecules trapped within aggregates of proteins that saturate the membrane. In cortical membranes, the amount of membrane proteins is significantly smaller and, thus, mainly induces formation of boundary lipids, which ASL cannot discriminate.

3.3. Saturation recovery curves complement discrimination of different lipid environments in intact membranes

We applied the DOT method using *n*-SASL and ASL to discriminate membrane domains in intact membranes and using n-PC, T-PC, ASL, and CSL to discriminate membrane domains in lens lipid membranes (Subczynski et al., 2007). Representative SR signals are shown in Fig. 5 for nuclear lens lipid and intact membranes. As can be seen in the figure, the SR signals of 5-SASL (Fig. 5B) and ASL (Fig. 5D) were successfully fit only with double exponential functions, both in the presence and absence of oxygen. This indicates that in both, cortical and nuclear intact membranes, spin labels are located in two environments with different OTPs and also different fluidities. The SR signal of the spin label without oxygen is a measure of the spin-lattice relaxation time of the spin labels, which is a sensitive monitor of membrane fluidity (Mainali et al., 2011a). SR signals from other *n*-SASLs

applied for intact membranes can be fitted with two exponentials in both the presence of oxygen and in its absence (signals not shown). Thus, two lipid environments are detected, with different fluidities and different OTPs at all depths in the bilayer portion of the intact membranes. Greater OTP values and shorter spin-lattice relaxation times were assigned to the bulk and boundary lipids. Remaining values characterize another domain, called the *slow oxygen transport (SLOT)* domain in earlier papers (Ashikawa et al., 1994), which is formed by lipids in contact with two proteins and/or by lipids in contact with proteins and boundary lipids (trapped lipids). Both of these domains are discriminated in cortical and nuclear membranes.

The SR signals of all phospholipid-type spin labels in cortical and nuclear lens lipid membranes, both in the presence and absence of oxygen, can be successfully fit only with single-exponential functions (see representative SR signals for 5-PC in Fig. 5A). This finding indicates the presence of a single homogenous domain in cortical and nuclear lens lipid membranes: the bulk phospholipid-cholesterol domain. We have previously investigated in detail the properties of this domain in lens lipid membranes from different animals (Mainali et al., 2011b; Raguz et al., 2008; Raguz et al., 2009; Widomska et al., 2007a).

As reported earlier (Raguz et al., 2009) and confirmed here (Fig. 5C), the DOT method with ASL allows discrimination of the CBD, which coexists with the bulk phospholipid-cholesterol domain, in nuclear lens lipid membranes (with extremely low OTP values). SR signals of ASL discriminate two domains in intact membranes (Fig. 5D): one with an OTP close to that of 10-SASL in the bulk plus boundary domain and one with an OTP close to that in the SLOT domain.

3.4. Fluidity profiles across domains of intact cortical and nuclear membranes

Figures 6B and D summarize the results presented in section 3.1, showing profiles of the maximum-splitting values obtained from *n*-SASL located in different membrane domains of cortical and nuclear intact membranes. In each membrane, profiles across two domains are presented: one a more fluid domain and the other very rigid. Comparison of these profiles with those obtained across cortical and nuclear lens lipid membranes (Figs. 6A, C) indicates that profiles across more fluid domains are very similar to those of lens lipid membranes. We assigned this domain as a bulk lipid domain, which appears to be very weakly affected by the presence of membrane proteins. In the intact nuclear membrane, this domain can be discriminated only with 12- and 16-SASL.

The profile of the maximum-splitting value across the second domain, which is formed as a result of the interaction of lipids with membrane proteins, indicates that lipids in this domain are strongly immobilized. Maximum splitting measured in the center of this domain in both cortical and nuclear intact membranes was close to the rigid limit value for 16-SASL. Thus, the ends of the alkyl chains of these molecules are completely immobile (in the time scale of the conventional EPR spin-label approach). Bell-shaped profiles of membrane fluidity in these domains contrast those in bulk lipids, which show lower fluidity in the center. Thus, alkyl chains of phospholipid molecules that are in contact with membrane proteins—both boundary and trapped phospholipids—are strongly immobilized but show higher mobility close to their headgroups. The gradient of fluidity toward the membrane surface is even greater than that expected from Fig. 6D because the rigid limit values for *n*-SASL also increase toward the membrane surface (see Fig. 9D, remembering that the rigid limit value is the $2A_Z$ value).

3.5. Profiles of the OTP across domains of intact cortical and nuclear membranes

Profiles of the OTP (Figs. 7B, D) complement the discrimination process for membrane domains in intact cortical and nuclear membranes. As indicated earlier (Ashikawa et al., 1994), the OTP cannot discriminate between bulk and boundary lipids because of the rapid lipid exchange between these domains (Ryba et al., 1987). Here, profiles across the bulk plus boundary cortical (Fig. 7B) and nuclear (Fig. 7D) domains show OTP values that are 30 to 40% smaller than OTP values for profiles across cortical (Fig. 7A) and nuclear (Fig. 7C) lens lipid membranes. This is only true in the membrane center (deeper than the C9 position). Close to the membrane surface the effect of membrane proteins is rather negligible. This may be due to the presence of saturating amounts of cholesterol, which strongly decreases oxygen transport to the depth of C9. These changes make the shape of the OTP profile across the bulk plus boundary domain in intact membranes bell-shaped, with a gradual increase in the OTP from the membrane surface to its center. These profiles contrast with the rectangular shape of OTP profiles in lens lipid membranes (Figs. 7A and C).

Values of the OTP in the center of the bulk plus boundary lipid domain of the nuclear intact membrane (Fig. 7D) are about 25% smaller than OTP values in the center of the bulk plus boundary lipid domain of the cortical intact membrane (Fig. 7B). This difference is likely the result of the greater protein content in nuclear membranes. Thus, the relative amount of boundary lipids compared to bulk lipids should be larger in intact nuclear membranes than in cortical membranes.

Profiles of the OTP across the domain formed by trapped lipids (the SLOT domain) are shown in Fig. 7B for cortical membranes and in Fig. 7D for nuclear intact membranes. Values of the OTP in these domains are ~10 times lower than in water and ~20 times lower than in the center of the bulk plus boundary domain. Thus, across this domain, the resistance to oxygen transport should be great. Based on these profiles and methods described in detail previously (Widomska et al., 2007b), we evaluated the membrane permeability coefficient for oxygen across all investigated domains (Table 1). Values of the OTP obtained with ASL and CSL were included in the profiles at appropriate depths (see (Raguz et al., 2008) for depth evaluation). This allowed us to draw a profile of the OTP across the CBD in nuclear lens lipid membranes (Fig. 7C). Also, ASL discriminated two domains in intact membranes, giving two values for the OTP. These values fit into the profiles obtained for *n*-SASL in intact membranes (Fig. 7B, D). We expected that ASL could discriminate the CBD domain in intact nuclear membranes, as it does in nuclear lens lipid membranes (Fig. 7C). However, values of the OTP in the CBD are very close to those in the trapped lipid domain (Fig. 7D), which makes discrimination between these two domains impossible. It must be remembered that OTP determines membrane fluidity differently from maximum splitting, order parameter, or spin-lattice relaxation time. In the first case, membrane fluidity is determined by the translational diffusion of oxygen within the lipid bilayer. The last three cases reflect the order and dynamics of alkyl chains.

3.6. Hydrophobicity profiles across intact membranes

Figure 8 contains representative EPR spectra of lipid spin labels in a frozen solution of lens lipid and intact membranes and shows the method of measuring $2A_Z$ values. Smaller $2A_Z$ values indicate higher hydrophobicity. Thus, smaller $2A_Z$ values from the spectra of 16-SASL and 16-PC in intact and lens lipid membranes compared with those of 5-SASL and 5-PC indicate that the membrane interior is more hydrophobic than the region close to the membrane surface.

Figures 9B, D show averaged hydrophobicity profiles across the lipid bilayer portion of the cortical and nuclear intact membranes. Both hydrophobicity profiles show a similar bell

shape with similar $2A_Z$ values. The $2A_Z$ values in the center of both membranes (14- and 16-SASL) indicate that hydrophobicity in this region can be compared to that of N-butylamine and 1-decanol ($\epsilon = 6-8$), and hydrophobicity near the membrane surface (5- and 7-SASL) can be compared to that of methanol ($\epsilon \sim 35$) (although this is still considerably less polar than in the aqueous phase with $\epsilon = 80$). For brevity, we relate the local hydrophobicity as observed by $2A_Z$ values to the hydrophobicity (or ϵ) of the bulk organic solvent by referring to Fig. 2 in (Subczynski et al., 1994). Thus, both profiles indicate the existence of a high hydrophobic barrier in intact lens membranes.

Figures 9A, D also contain hydrophobicity profiles across cortical and nuclear lens lipid membranes. Both profiles show a similar rectangular shape with an abrupt increase of hydrophobicity between C9 and C10. The $2A_Z$ values in the center of these profiles (10-, 12-, 14-, and 16-PC) indicate that hydrophobicity in this region is comparable to that of hexane and dipropylamine ($\epsilon = 2-3$), and hydrophobicity near the membrane surface (5- and 7-PC) can be compared to that of methanol ($\epsilon \sim 35$).

The presence of proteins changes a rectangular hydrophobicity profile (as observed for lens lipid membranes [Figs. 9A, C]) to one that is bell-shaped (Figs. 9B, D). The center of intact membranes becomes less hydrophobic than that of lens lipid membranes. Similarly, as in profiles of the OTP, the effect of membrane proteins on hydrophobicity profiles close to the membrane surface is rather negligible. Because $2A_Z$ values were measured for a frozen membrane suspension, the EPR spectrum is the superposition of individual spectra from all domains.

4. Discussion

The very high protein content in fiber cell plasma membranes should greatly affect the organization of lipids in intact membranes. Indeed, conventional EPR spectra and SR signals of lipid spin labels differ drastically from those recorded for lens lipid membranes (Figs. 3–5). All of these differences are considered to be mainly due to the effect of membrane proteins, which alter the lateral organization and dynamics of the lipid bilayer portion of the fiber cell plasma membrane. With each EPR method, two domains were detected for both cortical and nuclear intact membranes. Because of their different time scales, these methods discriminate different lipid environments, allowing us to obtain a more detailed picture of lateral lipid organization. Using conventional EPR, we were able to discriminate bulk lipids (with properties similar to those in bulk lipid domains in lens lipid membranes) from other more immobilized lipids (boundary lipids plus trapped lipids). The second lipid environment is likely due to lipid-protein interaction. The SR-EPR DOT method allowed us to discriminate the bulk plus boundary lipids from the trapped lipids. Thus, we can conclude that in both cortical and nuclear intact membranes, three types of lipid domains exist: the bulk lipid domain, which contains lipids nearly unaffected by the presence of membrane proteins; the boundary lipid domain, which contains lipids in contact with protein and bulk lipids; and the trapped lipid domain (SLOT domain), which contains lipids in contact with two proteins and/or lipids in contact with protein and boundary lipids.

AQP0, Cx46, and Cx50 are good candidates to order (the boundary lipid domain) and/or trap (the SLOT domain) lipids in fiber cell membranes. It is known that each of these specific proteins forms ordered two-dimensional arrays in fiber cell membranes (Buzhynskyy et al., 2007; Costello et al., 1989; Dunia et al., 2006; Zampighi et al., 2002). Thus, lipids can be trapped within these protein-dense structures forming SLOT domains similar to those formed by bacteriorhodopsin in the purple membranes of *Halobacterium halobium* (Ashikawa et al., 1994). Kuszak et al. (Kuszak et al., 2004) indicated that the density/distribution of these proteins is species-specific and varies along fiber length and as

a function of fiber depth (age). Indeed, it has been shown by Costello et al. (Costello et al., 1989) that the ordered arrays of AQP0 are enriched in the nucleus. This finding is consistent with our current data, which show that the amount of trapped lipid and the extent of lipid rigidity are greater in the nucleus than in the cortex. We can conclude that both the amount of protein and the packing of protein in membranes from the nucleus might be important in modifying the observed lipid physical properties. Moreover, Kuszak and Costello (Kuszak and Costello, 2004) suggest that in addition to channel functions fiber cell membranes have specialized AQP0-lipid interactions that determine the cell shape, surface topology, intercellular junctions, and important features of membrane aging and cataract formation.

Interestingly, Sato et al. (Sato et al., 1996) and Zhang et al. (Zhang et al., 1999) showed that the structural order determined by the static measure of the *trans/gauche* rotamer ratio in the hydrocarbon chains of bovine and human lens membranes is not affected by the presence of intrinsic and extrinsic lens proteins. These results are not in contrast with our results. The structural order describes the bulk membrane properties without discrimination of membrane domains and without showing depth dependences. Molecular order of hydrocarbon chains measured with the conventional EPR spin-labeling method can be obtained in different membrane domains as a function of depth. Moreover, the SR-EPR DOT method gives information about the fluidity of different membrane domains as a function of membrane depth. These static and dynamic descriptions of membrane properties complement each other, allowing us to conclude that the drastic changes in the structure and dynamics of the lipid-bilayer portion of the fiber cell plasma membrane induced by the presence of membrane proteins do not affect the total number of *gauche* rotamers in hydrocarbon chains.

Because of the difficulties of incorporating the lipid spin label at the polar headgroup in the intact membrane, we were not able to monitor how a very high protein content in fiber cell plasma membranes affects the organization and mobility of the polar headgroups. The literature indicates that the extrinsic membrane protein α -crystallin (when bound to sphingomyelin liposomes) can decrease or increase the flexibility of the sphingomyelin headgroup, depending on its structure (Tang and Borchman, 1998). More data about the interaction of membrane lipids with integral transmembrane and peripheral surface-binding proteins can be found in recent reviews (Marsh, 2008, 2010).

The interior of intact membranes was found to be less hydrophobic than the interior of lens lipid membranes. However, intact membranes still formed a high hydrophobic barrier to the movement of polar molecules into and out of fiber cells. The effect of integral proteins on the hydrophobicity of their lipid environment is complex and strongly depends on the amino acid composition of the membrane surface. It was shown that some transmembrane peptides increase membrane hydrophobicity (Subczynski et al., 1998; Subczynski et al., 2003), while others decrease the hydrophobicity of the membrane interior to as low as 2-propanol and ethanol with $\epsilon = 18\text{--}25$ (Ge and Freed, 1993; Wisniewska and Subczynski, 1996).

Most significantly, we found that the high cholesterol and protein contents in fiber cell plasma membranes strongly decrease the oxygen permeability across the lipid bilayer portion of these membranes. Cholesterol decreases oxygen permeability across the bulk lipid domain (Widomska et al., 2007b) and induces formation of the CBD with a very low oxygen permeability coefficient (Raguz et al., 2008; Raguz et al., 2009). Proteins induce formation of boundary lipids, which by “mixing” with bulk lipids additionally decrease the oxygen permeability coefficient across the bulk plus boundary domain (see (Ashikawa et al., 1994) and Table 1). Oxygen permeability across the trapped lipid domain, which is great in nuclear intact membranes, is even smaller than that across the CBD (see (Ashikawa et al., 1994) and Table 1).

Because proteins are nearly impermeable to oxygen (Altenbach et al., 1994; Subczynski et al., 1992b), they effectively alter the permeability coefficient for oxygen across the fiber cell plasma membrane. The effective permeability coefficient for oxygen is equal to the permeability coefficient for oxygen evaluated for the lipid bilayer portion of the membrane (shown in Table 1), multiplied by a factor proportional to the surface area of the lipid bilayer portion, and divided by the surface area of the entire membrane. Keeping in mind the statements above, the evaluations summarized in Table 1, and the fact that nuclear membranes have a greater number of membrane proteins than cortical membranes (Jacob et al., 1999), we can conclude that nuclear fiber cell membranes form greater barriers to oxygen permeation than cortical membranes.

Currently, we can state with great confidence that fiber cell plasma membranes form a significant barrier to oxygen transport. Because oxygen must pass through a few thousand fiber cell membranes on its way to the lens center, this barrier should help to maintain a low oxygen concentration in the eye lens interior. All results indicate that fiber cell plasma membranes become more rigid and less permeable to oxygen with age.

The results reported here complement our studies on the organization and dynamics of cortical and nuclear lens lipid membranes derived from two-year-old cows (Raguz et al., 2009). The order parameter, OTP, and hydrophobicity across cortical and nuclear porcine lens lipid membranes are nearly identical and very similar to those across cortical and nuclear bovine lens lipid membranes. These profiles were also similar to profiles across membranes derived from the lens lipids of six-month-old calves and pigs (Raguz et al., 2008; Widomska et al., 2007a; Widomska et al., 2007b).

The data presented here further support our hypothesis that a high cholesterol content keeps the bulk physical properties of lens lipid membranes relatively constant, even with age-related changes in phospholipid composition (the phospholipid composition of cortical and nuclear membranes differs significantly, which also reflects age-related changes). The presence of the CBD ensures that the surrounding phospholipid bilayer is saturated with cholesterol. These data provide improved understanding of the function of cholesterol and the CBD in eye lens membranes.

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- Physical properties of intact cortical and nuclear fiber cell plasma membranes
- Discriminated domains are bulk lipid, boundary lipid, and trapped lipid domains
- Formation of rigid and practically impermeable domains is enhanced in the nucleus

Fig. 1. Chemical structures of phospholipid- (*n*-PCs, T-PC, *n*-SASLs) and cholesterol-type spin labels (CSL, ASL) together with the structure of POPC (the most abundant phospholipid in porcine lens membranes) and cholesterol (Chol). Approximate locations of these molecules across the lipid bilayer membrane are illustrated.

Fig. 2.

Schematic drawing of (A) the cortical lens lipid membrane, which is formed by bulk lipids; (B) the cortical intact membrane, which contains bulk plus boundary and trapped lipids; (C) the nuclear lens lipid membrane with the pure CBD within the bulk lipids; and (D) the nuclear intact membrane, which contains bulk plus boundary, trapped lipids, and the CBD. Phospholipid spin labels are only located in the bulk lipid when it coexists with the pure CBD. However, spin-labeled cholesterol analogues are distributed between both regions (C). Black dots indicate the nitroxide moieties of spin labels.

Fig. 3. EPR spectra of 5- and 12-PC (A, C, E, and G) along with 5- and 12-SASL (B, D, F, and H) from lens lipid and intact membranes, respectively. Spectra A, B, E, and F are from cortical membranes; spectra C, D, G, and H are from nuclear membranes. Spectra were recorded at 37°C. Arrows 1–3 represent spectra from strongly immobilized, weakly immobilized, and water components, respectively. The measured values used to evaluate maximum splitting are indicated. The positions of certain peaks were evaluated with a high level of accuracy by monitoring them at 10 times higher receiver gain and, when necessary, a higher modulation amplitude.

Fig. 4. EPR spectra of ASL from lens lipid (A, C) and intact (B, D) membranes. Spectra A and B are from cortical membranes; C and D are from nuclear membranes. Spectra were recorded at 37°C. Arrows 1 and 2 represent spectra from strongly immobilized and weakly immobilized components, respectively. The positions of certain peaks were evaluated with a high level of accuracy by monitoring them at 10 times higher receiver gain and, when necessary, a higher modulation amplitude.

Fig. 5.

Representative SR signals with fitted curves and residuals (the experimental signal minus the fitted curve) for phospholipid analogues spin labels (5-PC for nuclear lens lipids and 5-SASL for nuclear intact membranes) and cholesterol analogues spin labels (ASL for both nuclear lens lipids and nuclear intact membranes) are shown. Signals were recorded for samples equilibrated with 100% nitrogen (A–D) and a gas mixture of 50% air/50% nitrogen (A–D). For nuclear lens lipid membranes, the SR signal in the presence of 100% nitrogen can be satisfactorily fitted with a single exponential function: for 5-PC, with a time constant of (A) $3.72 \pm 0.01 \mu$ s, and for ASL, with a time constant of (C) $3.03 \pm 0.01 \mu$ s. Upper residuals are for single-exponential fits. However, for nuclear intact membranes, the SR signal in the presence of 100% nitrogen can be satisfactorily fitted with only a double-exponential function: for 5-SASL, with time constants of (B) $6.1 \pm 0.20 \mu$ s and $2.53 \pm 0.04 \mu$ s, and for ASL, with time constants of (D) $6.21 \pm 0.30 \mu$ s and $2.02 \pm 0.09 \mu$ s. The first residual is for single- and the second residual for double-exponential fits. Again, for nuclear

lens lipid membranes, the SR signal in the presence of molecular oxygen can be satisfactorily fitted with a single-exponential function only for 5-PC with a time constant of (A) $1.85 \pm 0.01 \mu\text{s}$ (lower residual is for single-exponential fit) and with double-exponential curves for ASL with time constants of (C) $1.93 \pm 0.10 \mu\text{s}$ and $0.55 \pm 0.01 \mu\text{s}$ (the middle residual is for single- and the lower residual for double-exponential fits). However, for nuclear intact membranes, the SR signal in the presence of molecular oxygen can be fitted satisfactorily only with a double-exponential function: for 5-SASL, with time constants of (B) $4.14 \pm 0.20 \mu\text{s}$ and $1.54 \pm 0.04 \mu\text{s}$, and for ASL, with time constants of (D) $3.51 \pm 0.14 \mu\text{s}$ and $0.75 \pm 0.02 \mu\text{s}$. The third residual is for single- and the fourth residual for double-exponential fits.

Fig. 6. Profiles of maximum-splitting were obtained with *n*-PCs and *n*-SASLs at 37°C for (A) cortical lens lipid membranes, (B) cortical intact membranes, (C) nuclear lens lipid membranes, (D) and nuclear intact membrane. Schemes for membrane structures are shown in Fig. 2. Approximate localizations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 7.

Profiles of the OTP for (A) cortical lens lipid membranes, (B) cortical intact membranes, (C) nuclear lens lipid membranes, and (D) nuclear intact membranes at 37°C. Schemes for membrane structures are shown in Fig. 2. The saturating cholesterol concentration is responsible for the rectangular shape of the profiles in the bulk lipids of lens lipid membranes, whereas proteins are responsible for the bell shape of profiles in the bulk, plus boundary, lipids of intact membranes. Approximate localizations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 8. EPR spectra of 5- and 16-PC of nuclear lens lipid membranes (A, C) along with the EPR spectra of 5- and 16-SASL of nuclear intact membranes (B, D). Spectra were recorded at -165°C to cancel motional effects. The measured $2A_z$ value is indicated.

Fig. 9. Profiles of hydrophobicity ($2A_Z$) for (A) cortical lens lipid membranes, (B) cortical intact membranes, (C) nuclear lens lipid membranes, and (D) nuclear intact membrane at -165°C . Schemes for membrane structures are shown in Fig. 2. The rectangular shape is characteristic of lens lipid membranes, whereas the bell shape is characteristic of intact membranes. Approximate localizations of the nitroxide moieties of spin labels are indicated by arrows.

Table 1

Permeability coefficients for oxygen (P_M) across the hydrocarbon region of lens lipid and intact membranes at 37°C

	P_M (cm/s)
Cortical lens lipid membranes (Bulk lipid)	108.01
Nuclear lens lipid membranes (Bulk lipid)	84.40
Nuclear lens lipid membranes (CBD)	36.62
Cortical intact membranes (Bulk+Boundary lipid)	96.06
Nuclear intact membranes (Bulk+Boundary lipid)	68.61
Cortical intact membranes (Trapped lipid)	13.18
Nuclear intact membranes (Trapped lipid)	13.19
Water layer*	94.38

* The thickness of the water layer is the same as the hydrocarbon region.